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MINIREVIEW

Critical Analysis of Compositions and Protective Efficacies of Oral Killed Cholera Vaccines

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Two cholera vaccines, sold as Shanchol and Dukoral, are currently available. This review presents a critical analysis of the protective efficacies of these vaccines. Children under 5 years of age are very vulnerable to cholera and account for the highest incidence of cholera cases and more than half of the resulting deaths. Both Shanchol and Dukoral are two-spaced-dose oral vaccines comprising large numbers of killed cholera bacteria. The former contains *Vibrio cholerae* O1 and O139 cells, and the latter contains *V. cholerae* O1 cells with the recombinant B subunit of cholera toxin. In a field trial in Kolkata (India), Shanchol, the preferred vaccine, protected 45% of the test subjects in all of the age groups and only 17% of the children under 5 years of age during the first year of surveillance. In a field trial in Peru, two spaced doses of Dukoral offered negative protection in children under 5 years of age and little protection (15%) in vaccinees over 6 years of age during the first year of surveillance. Little is known about Dukoral's long-term protective efficacy. Both of these vaccines have questionable compositions, using *V. cholerae* O1 strains isolated in 1947 that have been inactivated by heat and formalin treatments that may denature protein. Immunological studies revealed Dukoral's reduced and short-lived efficacy, as measured by several immunological endpoints. Various factors, such as the necessity for multiple doses, poor protection of children under 5 years of age, the requirement of a cold supply chain, production costs, and complex logistics of vaccine delivery, greatly reduce the suitability of either of these vaccines for endemic or epidemic cholera control in resource-poor settings.

Cholera is an acute intestinal infection caused by the Gram-negative bacterium *Vibrio cholerae*, which colonizes the small intestine without invading the epithelium. Ingestion of food and/or drinking water contaminated with *V. cholerae* can cause the disease, which is often mild or asymptomatic but can sometimes be severe. The disease, which affects only humans, is mediated by cholera toxin (CT), which is secreted by *V. cholerae* in the intestine and acts upon the mucosal cells of the gut, causing a copious, painless, watery diarrhea that can lead to severe dehydration and shock. If it is left untreated, death can occur within hours. Cholera, a social disease arising out of poverty and a lack of basic sanitation, currently prevails in parts of Asia, Africa, and Latin America. Although cholera outbreaks have occurred in Europe and the United States, the disease has been essentially eradicated there through effective sanitation and public health measures (1).

Although more than 200 serogroups of *V. cholerae* have been identified, most cases of cholera are caused by two serogoups, O1 and O139 (2). *V. cholerae* O1 has two biotypes (classical and El Tor), each of which is further subdivided into two serotypes (Ogawa and Inaba). *V. cholerae* O1 and O139 secrete similar CTs, but they differ in the composition of their surface components, as *V. cholerae* O139 produces a polysaccharide capsule (3, 4). Hence, previous exposure to *V. cholerae* O1 does not confer immunity to attacks by *V. cholerae* O139. Outbreaks due to *V. cholerae* O139 occurred first in India in 1992 and then in neighboring countries in the following years but have been rarely reported during the last decade (5, 6).

Antibodies to various cholera antigens, such as lipopolysaccharide (LPS), outer membrane proteins, CT, and the major subunit of the toxin-coregulated pilus (TcpA), have been detected in serum samples from individuals immunized with *V. cholerae* O1 or from convalescent patients (7–11). *V. cholerae* O1 infection in cholera patients induces both memory B and T cell responses (12–14). Although intestinal lavage and human blood have been used to study immune responses, these materials may not correspond to the actual level of immunoglobulins in the gut after an antigenic stimulus (15, 16). Ethical considerations can limit a detailed investigation of the immune responses that occur in the guts of cholera patients. However, a thorough study of immune responses is possible in experimental animals such as rabbits (17, 18). A single-dose intraduodenal inoculation of live *V. cholerae* O1 into rabbits produced antibodies to both somatic (LPS and cell surface proteins) and secreted (CT and neuraminidase) antigens in various body fluids (serum and bile) and intestinal extracts from rabbits, the latter containing predominantly IgA together with a considerable amount of IgG (18). A study in the United States with volunteers who were orally immunized and subsequently challenged with live *V. cholerae* O1 demonstrated that cholera infection can induce a high degree of protection for up to 3 years against a challenge with either the Ogawa or the Inaba serotype of the same biotype (19).

CHOLERA VACCINES

In 1883, Robert Koch identified *V. cholerae* O1 as the etiological agent of cholera (20). Soon afterward, parenteral cholera vaccines were used in humans by the Spanish physician Ferran, who also introduced the concept of mass oral immunization with live *V. cholerae* O1 through drinking water supplies (21). Parenteral killed cholera vaccines were used from then until the 1970s and afterward discarded, as these vaccines offered protection for a lim-

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TABLE 1 Composition of killed whole-cell OCV Shanchol[a]

No. of cells	No. of EU[b] of LPS	Inactivation method	Strain/serotype	Biotype	Other
2.5×10^{10}	300	Heat	O1/Inaba	Classic	Cairo 48
5.0×10^{10}	600	Formaldehyde	O1/Inaba	El Tor	Phil 6973
2.5×10^{10}	300	Heat	O1/Ogawa	Classic	Cairo 50
2.5×10^{10}	300	Formaldehyde	O1/Ogawa	Classic	Cairo 50
5.0×10^{10}	600	Formaldehyde	O139		4260B

[a] Each 1.5-ml oral dose contains a 1.25×10^{11} O1 and 5×10^{10} O139 *Vibrio* cells, not more than 0.02% (wt/vol) thimerosal, and buffer *quantum satis* to 1.5 ml. The information shown is from references 28, 32, 37, and 41.
[b] EU, ELISA units.

ited duration, lasting up to only 6 months (22). Since the 1980s, oral cholera vaccines (OCVs) comprising either killed or live cells have been used. Of these, the following oral vaccines have been subjected to large-scale field trials: killed whole *V. cholerae* O1 cells (WC), WC with the B subunit of CT (CTB) isolated from culture supernatant (WC-CTB), WC with the CTB prepared by recombinant DNA technology (WC-rCTB), WC with killed *V. cholerae* O139 cells and live attenuated *V. cholerae* O1 cells (23–30). Only two vaccines, WC-rCTB and WC with *V. cholerae* O139, sold commercially as Dukoral and Shanchol, respectively, are currently available and have been prequalified by the World Health Organization (WHO) (31, 32). As cholera is a public health problem for poor people in various regions of the world, a critical analysis of the compositions and protective efficacies of the currently available OCVs is essential, as many questions related to their compositions and protective efficacies remain unanswered. Of these two vaccines, Shanchol is now preferred for mass vaccination against cholera (33–35) and will be discussed first. There are an estimated 3 to 5 million cholera cases and 100,000 to 120,000 deaths worldwide per year, with children under 5 years of age accounting for the highest incidence of cholera and more than half of the deaths (36). Results presented in this review demonstrate that neither Shanchol nor Dukoral can offer effective protection against endemic or epidemic cholera, especially among children under 5 years of age, the group most vulnerable to cholera.

SHANCHOL

Shanchol is the trade name of a candidate OCV comprising large amounts of two groups of killed cholera bacteria (*V. cholerae* O1 and O139). It is manufactured by Shantha Biotechnics of India, a subsidiary of the French pharmaceutical company Sanofi-Aventis (28, 29, 32). It is a two-dose oral vaccine to be taken with a minimum interval of 2 weeks; immunity against cholera is expected to appear 7 to 10 days after the second dose (28, 32). This vaccine has been developed primarily by a group of scientists from Sweden and South Korea, and its initial studies were carried out in Vietnam (37). As the national regulatory authority of Vietnam was not recognized by the WHO, the study was continued in India, since that country's regulatory authority meets the WHO requirements for global marketing (28). In 2006, the vaccine was subjected to a large-scale field trial in an impoverished area of Kolkata (India) where cholera is endemic, and the results of the vaccine's performance during the subsequent years have been published (23, 28, 29). The WHO prequalified the vaccine on 29 September 2011 (32).

A cholera epidemic caused by *V. cholerae* O1 (El Tor, Ogawa) struck Haiti in October 2010 with catastrophic consequences, claiming 8,546 lives and sickening more than 700,541 people in the first 3 years and 5 months (i.e., through 10 March 2014) (38, 39). Shanchol was used in 2012 in a pilot study to demonstrate the feasibility of mass vaccination in urban and rural Haiti (33, 34). As the study was not aimed to monitor Shanchol's effectiveness, information on its protective efficacy against cholera in the Haitian population remains unknown. Although the future use of this two-dose vaccine has been proposed (33–35), concerns about the operational and logistic challenges regarding its deployment in cholera outbreaks have been raised (40). Several aspects of this vaccine related to its composition and protective efficacy require in-depth examination.

COMPOSITION OF SHANCHOL

Shanchol comprises four strains, one of *V. cholerae* O139 (4260B) and three of *V. cholerae* O1 (two classical [Cairo 48, Cairo 50] and one El Tor [Phil 6973]). All are killed, some by heating and some by formaldehyde treatment (28, 32, 37, 41) (Table 1). The vaccine contains a total of 1.75×10^{11} cells (7.5×10^{10} cells of the classical O1 strains, 5.0×10^{10} cells of the El Tor strain, and 5.0×10^{10} cells of the O139 strain). The *V. cholerae* strain content of the vaccine, claimed to be prepared in conformity with WHO standards, is expressed in enzyme-linked immunosorbent assay (ELISA) units of LPS (28, 32, 37). The vaccine is reported to contain 300 ELISA units of LPS of each of the three preparations of classical strains, 600 ELISA units of LPS of the El Tor strain, and 600 ELISA units of LPS of the O139 strain.

VACCINE PARTICIPANTS

This was a cluster randomized, double-blind, placebo-controlled trial carried out in 3,933 dwellings with a total population of 107,774 (28). However, about one-third of them ($n = 38{,}027$) declined to take part in the program. Two spaced doses of the vaccine were administered to 31,932 persons, excluding infants under the age of 1 year and pregnant women. A placebo of heat-killed *Escherichia coli* K-12 was fed to 34,968 persons. Of the two-dose vaccine recipients, 71% were >15 years of age, 22% were 5 to 15 years of age, and 7% were <5 years of age. Not all of the residents of the same dwelling took part in the trial.

PROTECTIVE EFFICACY

The trial recorded only severe cases of cholera requiring medical attention (23, 28). The vaccine's efficacy against asymptomatic and mild cases was not evaluated.

During the first year of surveillance, the vaccine protected 45% of those in all of the age groups but only 17% of the children under 5 years of age, the group most vulnerable to cholera (28, 29) (Table 2; Fig. 1). However, for unexplained reasons, the vaccine's protective efficacy rose sharply in the following

TABLE 2 Protective efficacy of OCV Shanchol by age and year of follow-up[a]

Age group (yr)	% Protective efficacy		
	Yr 1	Yr 2	Yr 3
<5	17	81	37
5–15	81	92	89
15+	66	62	64
All	45	77	65

[a] The data shown are based on information presented in reference 29.

year to 77% in all of the age groups and to 81% in children under 5 years of age (29). During the third year of surveillance, the vaccine's protective efficacy was 65 and 37% in all of the age groups and in children under 5 years of age, respectively (29). The vaccine protected 81, 92, and 89% of the older children 5 to 15 years old for surveillance periods of 1, 2, and 3 years, respectively (29). During the 4th and 5th years, the vaccine protected 58 and 80%, respectively, of those in all of the age groups. Separate data for these years in children under 5 years old, those 5 to 15 years old, and persons above 15 years old were not reported (23). The vaccine's cumulative protective efficacy during 5 years of surveillance was 65, 42, 68, and 74% in all of the age groups, children under 5 years old, those 5 to 15 years old, and participants above 15 years old, respectively (23).

CONCERNS REGARDING THE SHANCHOL VACCINE

There are several concerns regarding the vaccine's composition and protective efficacy and the reliability of the trial.

VACCINE COMPOSITION

The vaccine's composition has been described in ELISA units of LPS without defining what ELISA units of LPS are and how they were derived from killed *V. cholerae* cells (28, 32, 37, 41). It is well established that the two groups of *V. cholerae* (O1 and O139) have LPSs that differ in composition and amount (42, 43). *V. cholerae* O139 possesses both LPS and capsular polysaccharide (CPS), which are present at a ratio of 1:2, LPS being the minor component (43). *V. cholerae* O1 does not possess CPS. Although, the definition of an LPS unit in these vaccine strains is unclear, it is an interesting coincidence that 5.0×10^{10} cells of *V. cholerae*, whether of the O1 El Tor or O139 type, produced the same 600 ELISA units of LPS (28, 32, 41) (Table 1). Without a clear definition of the "ELISA units of LPS" used to quantitate the bacterial strains in the vaccine, it is extremely difficult to understand the vaccine's composition.

The rationale for strain selection and the method of killing (some strains with heat and some with formaldehyde) has not been provided. Importantly, the two classical strains (Cairo 48, Cairo 50) of the vaccine were collected from the Egyptian cholera epidemic of 1947. It is likely that these strains have undergone numerous transfers and variations since then. Further, information on the antigenic analysis of these strains after killing with heat or formaldehyde has not been provided, though it is known that these procedures can impact antigen expression.

Treatment with heat and formalin can denature the cell surface proteins of *V. cholerae* and alter the antigenic mosaic of its cells (44, 45). Formalin, a well-known cross-linking agent, can modify cell surface proteins of *V. cholerae* by reacting with primary amino groups to form unstable products that can react further with several other amino acid residues to form stable methylene bridges (46, 47). Besides, formalin treatment of proteins has been reported to constrain antigen presentation to T cells (46). Therefore, formalin treatment to produce a vaccine is less than ideal. A WC vaccine obtained by irradiation that destroyed only chromosomal DNA was reported to offer greater protection of rabbits against challenges than that offered by heat- or formalin-killed WC (45).

VACCINE'S EMPHASIS ON CHOLERA DUE TO CLASSICAL O1 STRAINS

The vaccine comprises three preparations of classical O1 strain preparations totaling 7.5×10^{10} cells and only one preparation of an El Tor strain containing 5.0×10^{10} cells (28, 32, 41) (Table 1). The cholera cases detected during the Kolkata trial and also in other recent epidemics such as those in Haiti and Zimbabwe were due to the El Tor biotype (28, 38, 48). The rationale for enriching the vaccine with classical strain preparations is not apparent and has not been provided.

V. CHOLERAE O139—A COMPONENT OF QUESTIONABLE VALUE

Although the vaccine contains a large proportion of killed *V. cholerae* O139 cells (29% of the total amount, 5.0×10^{10} cells), field trials in both India and Vietnam revealed that the *V. cholerae* O139 component of the vaccine induced weak immune responses; only 10% of the adults and 27% of the children under 18 seroconverted (37, 49). The vaccine's protective efficacy against cholera caused by *V. cholerae* O139 remains to be ascertained, as the cholera in Kolkata was caused by *V. cholerae* O1 (El Tor, Ogawa [28]). It is worth noting that cholera outbreaks due to *V. cholerae* O139 occurred mostly in southern Asia in the 1990s and have not been reported during the last decade (5, 6). Therefore, the inclusion of a large number (1×10^{11}) of killed *V. cholerae* O139 cells in two doses of the vaccine is of questionable value.

QUESTIONS REGARDING PROTECTIVE EFFICACY

More than 80% of the people infected with toxigenic *V. cholerae* do not develop any symptoms (50). A smaller proportion of

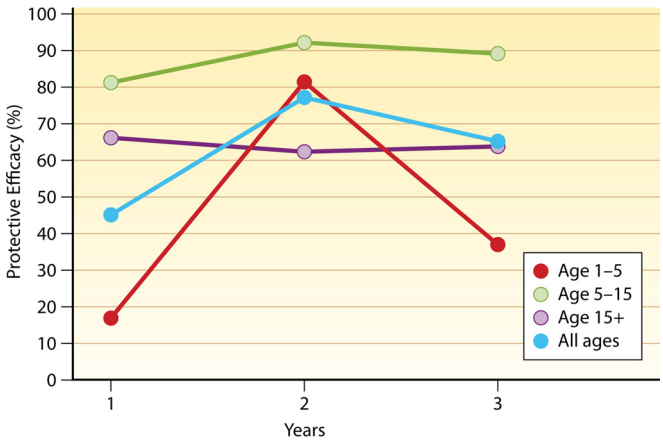

FIG 1 Performance of the OCV Shanchol by age and year of follow-up. Shown are the kinetics of the protective efficacy of the oral killed whole-cell cholera vaccine Shanchol among two-dose recipients in various age groups during the 3 years after the second dose (29).

symptomatic persons develop mild-to-moderate diarrhea, and only a small percentage develop severe cholera. As the Kolkata trial recorded only severe cases of cholera requiring medical attention, the vaccine's efficacy against asymptomatic and mild cases was not evaluated. Asymptomatic carriers can still shed bacteria and play a vital role in disseminating infection (51). The effect of Shanchol in reducing the incidence of carriers remains unknown.

Evaluating the protective efficacy of a cholera vaccine by carrying out the trial with an already primed adult population in an area where cholera is heavily endemic produces a biased picture of its efficacy (52). Knowing this, only children aged 0 to 14 were included in a few cholera vaccine trials in the 1960s in Bangladesh (53). But in the trial of Shanchol carried out in Kolkata, an area where cholera is heavily endemic, the vast majority (71%) of the participants were adults and older children above 15 with a high likelihood of exposure to cholera prior to vaccination (28). Therefore, results coming out of the Kolkata trial may not be applicable to the populations of other countries who have not been exposed to cholera antigens.

It is difficult to understand a mechanism by which the protective efficacy of a vaccine, monitored for several years, increases with time. However, in this trial, the protective efficacy of Shanchol was very poor (only 17%) in the most vulnerable group (i.e., children under 5 years of age) during the first year of surveillance (29). Remarkably, it climbed dramatically to 81% in the following year (29) (Table 2; Fig. 1). The vaccine's efficiency also increased from 45 to 77% in all of the age groups during the second year (28, 29). In contrast, during the 1985 OCV trial in Bangladesh, the protection of children under 5 years of age by the oral killed whole *V. cholerae* O1 cell (WC) vaccine progressively declined with time, with the protective efficacy being 31, 24, and 2% during the first, second, and third years, respectively (26). Compared to the Shanchol trial, even a single-dose parenteral classical bivalent (Ogawa and Inaba) whole-cell vaccine with aluminum adjuvant produced much better results in children under 5 years of age in the field trials carried out in India and Indonesia in the 1970s (54, 55). In a large-scale field trial in Kolkata in 1975, the parenteral vaccine with aluminum adjuvant protected 100% of the children under 5 years of age for 6 months, 89% of them for 12 months, and 92% of them for 18 months (54). The overall protection rate in all of the age groups during the 1-year surveillance period was 62%. Thus, the performance of two doses of Shanchol in the same city 30 years later was much inferior to that of the single-dose parenteral vaccine with aluminum adjuvant.

The protective efficacy of Shanchol in the Kolkata trial in older children 5 to 15 years old was very high (>80% during the 3 years of surveillance), even higher than that in adults, who because of exposure to cholera antigens in an area where cholera is endemic were expected to experience the best protection of all of the age groups (29) (Table 2; Fig. 1). Despite these highly unusual observations regarding protective efficacy, no credible explanation has been provided.

PROBLEMS WITH COST AND THE LOGISTICS OF DELIVERING THE VACCINE

Although the vaccine has been widely propagated as inexpensive (28, 29), it may not be economically feasible to deliver this vaccine to those who need it most, considering the fact that cholera is a social disease prevailing in resource-poor countries. The vaccine's negotiated price in 2011 with the manufacturer for bulk purchase (200,000 doses) for use in Haiti for a two-dose regimen was $3.70 (33). That did not include the cost involved in actually disseminating and administering it. Further, the vaccine has a cold supply chain requirement that is difficult to maintain in countries where cholera outbreaks occur. As it is a two-spaced-dose vaccine, the vaccination campaign encountered logistical and cold supply chain challenges involving substantial planning and has been described as "no small task" by the field workers delivering the vaccine in Haiti (33, 34). It is worthwhile to point out that the rationale for the requirement of a cold supply chain for Shanchol, a vaccine consisting largely of killed cells, has not been provided.

TABLE 3 Composition of WC-CTB/rCTB used in field trials[a]

No. of cells	Inactivation method	Serotype	Biotype	Strain
2.5×10^{10}	Heat	O1/Inaba	Classic	Cairo 48
2.5×10^{10}	Formaldehyde	O1/Inaba	El Tor	Phil 6973
2.5×10^{10}	Heat	O1/Ogawa	Classic	Cairo 50
2.5×10^{10}	Formaldehyde	O1/Ogawa	Classic	Cairo 50

[a] The data show the contents of one dose of WC and are from references 24 and 30. Each dose of the B subunit of cholera toxin contains 1 mg obtained either from the culture supernatant (CTB) or by recombinant technology (rCTB) from *V. cholerae* O1 (Inaba, classical). The vaccine was administered with a sodium bicarbonate buffer (24, 30).

CONFLICT OF INTEREST

Although the field trial was conducted by an institute of the Indian Government (The National Institute of Cholera and Enteric Diseases, Kolkata), with its director being the principal investigator of the program (28, 41), the confidentiality of the codes related to the trial was maintained by the private vaccine company Shantha Biotechnics and the vaccine's prime developer, the International Vaccine Institute (29). This introduced the potential for a conflict of interest. This could have been avoided if the trial had been monitored and its confidentiality maintained by an independent and impartial body with no conflict of interest or personal ties to those associated with the vaccine.

ORAL VACCINATION WITH A COMBINATION OF *V. CHOLERAE* O1 COMPONENTS

Killed WC-CTB vaccine's trial in Bangladesh. In 1976, two Swedish researchers reported that a combination of *V. cholerae* O1 antigens such as LPS and CT or choleragenoid (now termed CTB) induced more than 100-fold greater protection of rabbits against a challenge with live vibrios than did vaccination with either of the two antigens alone (56). To substantiate this claim, a vaccine (WC-CTB) comprising a combination of killed whole cholera bacteria (WC) and CTB was subjected to a large-scale, randomized, double blind, placebo-controlled field trial in Bangladesh in January 1985 (24). It is noteworthy that a Dutch study in 1987 with the same WC-CTB vaccine in rabbits could not reproduce the earlier claims made by the Swedish investigators (17). The WC-CTB vaccine comprised four different preparations of three *V. cholerae* O1 strains (Inaba [classical and El Tor] and Ogawa classical) and 1 mg of CTB isolated chemically from the culture supernatant produced by *V. cholerae* strain 569B (Inaba classical) (Table 3). All of the strains were killed, some with heat and some with formaldehyde treatment. The trial comprised 63,498 participants and included a control WC vaccine without CTB and a placebo of *E. coli* K-12. Each dose of the vaccine contained a total

TABLE 4 Protective efficacy of the WC-CTB vaccine during 3 years of surveillance of various age groups in Bangladesh[a]

Age group (yr)	% Protective efficacy			
	6 mo	1st yr	2nd yr	3rd yr
2–5	100	38	47	−37
>5	76	78	61	40
All	85	62	57	17

[a] The data shown are based on the publications cited in references 24 to 26 and 57.

TABLE 5 Protective efficacy of the WC vaccine during 3 years of surveillance of various age groups in Bangladesh[a]

Age group (yr)	% Protective efficacy			
	6 mo	1st yr	2nd yr	3rd yr
2–5	35	31	24	2
>5	71	67	73	62
All	58	53	57	43

[a] The data shown are based on the publications cited in references 24 to 26 and 57.

of 1×10^{11} cells (Table 3). Three spaced doses of the vaccine totaling 3×10^{11} cells were fed orally to each vaccine recipient. As children are at higher risk for cholera, 62% of the trial participants were children aged 2 to 15 years, the remainder comprising only adult females (≥15 years old). All adult males and children under 2 years old were excluded.

Prior to the field trial in Bangladesh, a few healthy adults in the United States were immunized orally with three spaced doses of either WC-CTB or WC vaccine. They were challenged with live *V. cholerae* O1 after 5 weeks of immunization (8). Both of the vaccines had a moderate protective efficacy of approximately 60%.

The trial in Bangladesh that had started in January 1985 was followed by a 6-month pre-epidemic period (April to September 1985) during which the incidence of cholera was low and the WC-CTB vaccine protected 85% of those in all of the age groups (24) (Table 4; Fig. 2). With the arrival of a cholera epidemic afterward, the protective efficacy of WC-CTB in all of the age groups fell to 62% at 1 year (25, 26) (Table 4; Fig. 2). Upon analysis by age group, the protective efficacy of WC-CTB in children 2 to 5 years old at 1 year fell drastically to 38% (25, 26) (Table 4; Fig. 2). During the third year of surveillance, the protective efficacy of WC-CTB fell significantly in all of the age groups, in participants >5 years old, and in children 2 to 5 years old to 17, 40, and −37% (negative), respectively (26) (Table 4; Fig. 2). The WC-CTB vaccine offered hardly any protection in vaccinees during the fourth year (57) and is no longer being produced.

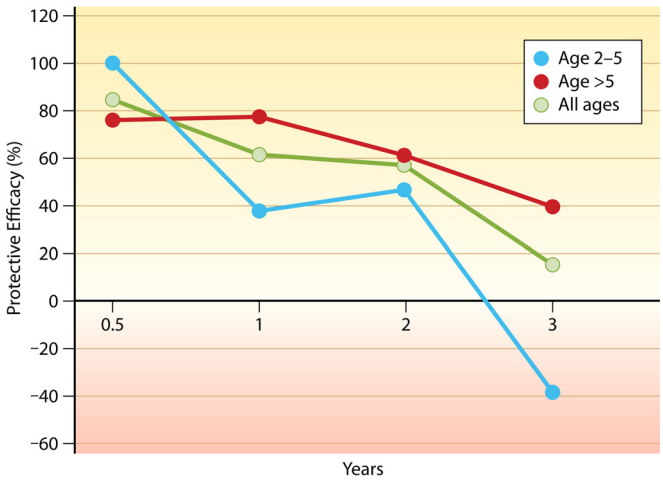

FIG 2 Performance of WC-CTB by age and year of follow-up. Shown are the kinetics of the protective efficacy offered by the oral killed whole-cell cholera vaccine (WC) with the CT B subunit (CTB, nonrecombinant) among three-dose recipients in various age groups in Bangladesh during the 3 years after the third dose (24–26, 57).

The protective efficacy of WC was lower (58%) than that of WC-CTB (85%) in all of the age groups during the initial 6 months after vaccination (24, 25) (Tables 4 and 5). During the first year, the protective efficacies of WC in all of the age groups, in participants >5 years old, and in children 2 to 5 years old were 53, 67, and 31%, respectively (26) (Table 5). While the protective efficacy of WC in participants above 5 years of age was in the range of 62 to 73% during the 3 years of follow-up, it was much lower (2 to 31%) in children 2 to 5 years old during that period and was not evident in the third year (26) (Table 5). Both of the vaccines enriched in *V. cholerae* O1 of the classical biotype were less protective against El Tor infections (25).

Antibody responses after immunization with WC-CTB and WC were evaluated in serum obtained from a number of randomly selected vaccinees (58). Two weeks after immunization, the geometric mean antitoxin titers were 2.5 to 4.5 times higher in vaccines who received the WC-CTB vaccine. The vibriocidal titers were 1.3 to 2.1 times higher in vaccinees who received both of the vaccines. However, this elevated vibriocidal titer persisted for only a brief period of time and was barely detectable after 7 months even though protection was observed afterward.

WC-RCTB VACCINE TRIAL

(i) South America. As the production of rCTB by DNA technology was first reported in 1989 (59), the WC-CTB vaccine's CTB unit was replaced in the early 1990s with rCTB prepared from *V. cholerae* O1 (classical) (60, 61). Subsequently, the WC-rCTB vaccine was marketed under the trade name Dukoral. A small-scale trial of WC-rCTB (Dukoral), carried out for a short period (18 weeks only) involving 1,426 military recruits in Peru in 1994 showed a protective efficacy against cholera of 86% (61). This relatively high protective efficacy was due to the occurrence of very few cases of cholera and the reassignment of the military recruits to other bases, which led to early closure of the trial (62). In 1994, WC-rCTB was subjected to a randomized, double-blind, placebo-controlled field trial in Peru, where participants (n = 17,799) received two spaced doses of either the vaccine (n = 9,012) or a placebo (n = 8,787) (30). During the first year of surveillance, the vaccine failed to protect vaccinees of any age (−4%). An analysis by age group showed that the vaccine did not protect children under 5 years of age and offered very little protection (15%) to recipients over 6 years of age. The protection level during the second year of surveillance increased only when a third booster dose was administered 10 months after the second one, with the protection being 61% in all of the age groups and 51.5% in children 2 to 5 years old. The trial was not continued beyond the second year. A debate took place afterward in which the viewpoints of those supporting Dukoral's two-dose regimen (63) were

rebutted by the scientists associated with the Peruvian trial (62). According to the proponents of the two-dose regimen, two doses of WC-CTB were as good as three doses, as claimed in the Bangladeshi trial of 1985 (26, 63). However, a recent study of OCVs conducted by the United Kingdom Cochrane Infectious Diseases Group has been unable to get access to the data to confirm this finding arising out of the Bangladeshi trial (64).

(ii) East Africa. In a field trial in 2009 that lasted only 14 months, 23,921 individuals (above 2 years of age) of Zanzibar, East Africa, were fed two spaced doses of WC-rCTB (65). The trial had several drawbacks. Instead of being a randomized, double blind, placebo-controlled study, the vaccine recipients were volunteers who had opted to receive the vaccine.

The controls, who did not receive the vaccine, differed in many ways from vaccine recipients. There were more males among the nonrecipients, who were older, drank more tap water, lived in more densely populated areas with lower neighborhood level vaccine coverage, and were less willing to take the vaccine. After 14 months of surveillance, the vaccine was reported to offer 79% protection against cholera in all of the participants. No data were presented on the vaccine's efficacy in children under the age of 5, the group most vulnerable to cholera. Moreover, the incidence of cholera was extremely low. Interestingly, the vaccine significantly increased the risk of noncholera diarrhea among the recipients, although an earlier study had suggested that the WC-CTB vaccine protected against enterotoxigenic *E. coli* diarrhea (66). The causes of noncholera diarrhea were not identified.

In a case-control trial of WC-rCTB in Mozambique in 2004, two doses of the vaccine were reported to offer 82 and 67% protection, respectively, for recipients below and above 5 years of age, respectively (67). Unfortunately, the trial was conducted for only 6 months, severely limiting any conclusions about Dukoral's protective efficacy.

LACK OF SYNERGY BETWEEN WC AND CTB/RCTB

Controlled trials of the WC-CTB/rCTB vaccine carried out in Bangladesh, Peru, and the United States (8, 24, 26, 30) failed to demonstrate any synergy between WC and CTB, as claimed earlier (56). The high protective efficacy of 85 to 86% offered by the WC-CTB/rCTB vaccine in Bangladesh and Peru, tacitly attributed to CTB/rCTB, was observed during the first 6 months and 18 weeks, respectively, when cholera cases were few. No differences in protective efficacy between the WC-CTB and WC vaccines were observed when the 1-year follow-up results in the Bangladeshi trial are considered, suggesting that antitoxic immunity played a short-term role and antibacterial immunity had a greater role in conferring longer protection. While the protective efficacies of these two vaccines in Bangladesh were moderate and similar during the second year of surveillance, the protective efficacy of the WC-CTB vaccine was inferior to that of the WC vaccine in the third year, affording negative protection of children 2 to 5 years old and even making them more susceptible to cholera (26, 68) (Fig. 2). It is worthwhile to point out that no long-term controlled field trial of WC-rCTB has been carried out, the Peruvian trial, lasting only 2 years, being the longest one.

CONCERNS REGARDING THE KILLED ORAL COMBINATION VACCINE (WC-CTB/RCTB)

(i) Strain selection and killing procedure. The *V. cholerae* O1 strains used for WC-CTB/rCTB vaccines are identical to those present in Shanchol (Table 3).These strains were isolated from the cholera epidemic in Egypt in 1947 and possibly underwent numerous transfers and variations since then. To produce the vaccine, some of the bacteria were killed with heat and some were killed with formaldehyde, and an antigenic analysis of these strains after such treatments has not been provided.

(ii) Requirement of a large amount of cells. A two-dose WC-rCTB vaccine used in field trials contained 2.00×10^{11} cells (30, 65), which is severalfold more than the number received by a vaccinee immunized with the now discarded killed parenteral WC vaccine containing 0.08×10^{11} cells/ml (68). This amount present in WC-rCTB is the equivalent of *V. cholerae* O1 growth from a confluently streaked petri dish (68). As of late 2011, the bacterial content of WC-rCTB has been further increased by 25%, to 1.25×10^{11} cells/dose, without providing a rationale for the increase (69).

(iii) Lack of information for controlling WC-RCTB. At present, there is no *in vitro* test to evaluate and compare the potencies of different lots of killed whole-cell OCVs (68, 70). This could account for the variation in the results obtained with WC-rCTB observed in different field trials (30, 65, 67).

B SUBUNIT OF CT IN WC-CTB/RCTB

CTB, incorporated in the WC-CTB/rCTB vaccine, is derived from a strain of the classical biotype (CT-1) that does not adequately protect against toxin produced by El Tor vibrios. This choice of the toxin B subunit is unfortunate, as almost all cholera is now caused by El Tor strains that produce CT-2, a B subunit related but not structurally or immunologically identical to CT-1 (71).

LIMITATIONS OF THE USE OF WC-RCTB

WC-CTB/rCTB is a two-spaced-dose vaccine with an interval of at least 1 week between doses. Immunity usually does not develop until 1 week after the second dose (60). The period between the initial vaccination and protective immunity would decrease efficacy during epidemics.

It cannot be given to children under the age of 2 despite the fact that they may be vulnerable to cholera (30, 60).

The vaccine's protective efficacy is short. For continuous protection against cholera, a single booster dose is recommended within 2 years for adults and children from 6 years of age and after 6 months for children aged 2 to 6 years (72). If more than 2 years have elapsed since the last vaccination, the primary vaccination course should be repeated (72).

The vaccine delivery system is inconvenient, requiring stomach acid neutralization, which can be problematic for people with stomach ailments (73).

The vaccine requires a large quantity of safe water, as the product is very voluminous (150 ml/dose) (60), 30 times more so than usual vaccines, thus limiting its application in recent epidemics (74, 75).

The vaccine's strict requirement of a cold supply chain and very high production cost make it unsuitable for use in many resource-poor countries where cholera prevails (73).

As stated by the manufacturer, formaldehyde used during the manufacturing process can be present in the final product and act as a potential allergen to those who are sensitive to it (76). Adverse reactions such as diarrhea, stomach cramps, vomiting, and fever have also been reported (77).

Cholera prevails in areas of Africa and Latin America also hit by the HIV/AIDS epidemic, but the vaccine is not recommended for

use by HIV-infected subjects, as it has been reported to increase HIV loads (from 2- to 60-fold) in the plasma of patients (72, 78).

IMMUNOLOGICAL STUDIES OF THE IMMUNE RESPONSE TO WC-RCTB

Recent humoral and cellular immunological studies of immune responses to *V. cholerae* O1 antigens in adults and children in Bangladesh comparing clinical infection and vaccination have provided a number of important observations (79–82). Vibriocidal antibodies, predominantly directed against LPS, are regarded as a measure of immunity (83). Children under 5 years of age who received two spaced doses of Dukoral (WC-rCTB) had (i) lower levels of antibodies to LPS, (ii) lower vibriocidal titers, and (iii) no memory B cell response to LPS, in contrast to children in the same age group with natural cholera infection (81). Adult vaccine recipients, while having anti-CTB and anti-LPS antibodies comparable to those detected in adult cholera patients, showed (i) weaker vibriocidal responses and (ii) no IgA or IgG memory B cell responses to LPS (79). Studies of antigen-specific memory T cell responses showed that cholera patients developed significant levels of toxin-specific memory T (T_{EM}) cells and cytokines characteristic of Th1, Th2, and Th17 cell responses (80). In contrast, younger children (2 to 6 years old) who received Dukoral neither developed T_{EM} nor showed an increase in Th1 cells but did show a decrease in Th17 cells and an increase in regulatory T cells, indicating diminished T cell memory responses required for the subsequent development of memory B cell responses. These findings may account for the lower protective efficacy of Dukoral in children under 5 years of age.

ON THE QUESTIONABLE BIOLOGICAL SIMILARITY BETWEEN CTB AND RCTB

The isolation of rCTB by recombinant DNA technology involves procedures that produce pure rCTB that is not contaminated with intact CT. Results with CTB prepared from two different sources may not necessarily be similar. CTB obtained by chemical purification of the cell-free supernatant may contain traces of CT, a very powerful adjuvant (84), that may escape detection by quality control assays but be sufficient to exert some of its biological functions. The substitution of rCTB for CTB can potentially weaken the vaccine. Some reports have correctly distinguished between WC-CTB and WC-rCTB (85, 86); however, the purity of chemically isolated products can vary from lot to lot, further confusing comparisons. Although a rise in serum antitoxin and vibriocidal antibody titers after oral immunization with WC-rCTB and WC-CTB was detected in a few Swedish volunteers, the mean vibriocidal antibody titer increase was greater in those who received WC-CTB (87). The WC-CTB vaccine used in the Swedish study was not from the same lot as that used in the Bangladeshi trial of 1985 (24). Volunteers in the Swedish study were not challenged with live *V. cholerae* O1, thereby providing no information on the comparative protective efficacies of these two vaccines (87, 88). To date, there have been no field trials simultaneously comparing the protective efficacies of WC-CTB and WC-rCTB. Therefore, the "practical" similarity between CTB and rCTB, as reported in the literature, is not supported by data (89, 90).

The cholera vaccine literature is replete with statements that the WC-rCTB vaccine (Dukoral) was subjected to a large-scale field trial in Bangladesh in 1985 offering 80 to 90% protection during the first 6 months after vaccination (91–96). For example, the manufacturer of Dukoral (SBL Vaccines AB Sweden) has used this statement for commercial purposes, describing the vaccine's protective efficacy to be 85% against cholera without specifying the period of duration (97). There is no record of a field trial of a vaccine containing WC-rCTB in Bangladesh in 1985. Further, since the production of rCTB by recombinant DNA technology was first reported in 1989 (59), reference to a field trial of WC-rCTB in 1985 is inaccurate. The Bangladeshi trial of 1985 used WC with CTB, and the latter was isolated biochemically from cell-free supernatants; it was not produced by recombinant technology (24). As stated earlier, assumptions that rCTB and CTB are essentially equivalent when incorporated into vaccines are not supported by data and it is critical to distinguish between vaccines that contain these different components. Consequently, using results from the 1985 Bangladeshi trial of WC-CTB to justify the use of a vaccine containing WC-rCTB is inappropriate and should be avoided. In the Bangladeshi trial, the WC-CTB vaccine had a protective efficacy of 85% in a large number of vaccinees of all ages ($n = 21{,}141$) during the initial 6 months (24). The WC-rCTB vaccine in Peru had a protective efficacy of 86% in a much smaller number of participants comprising healthy military recruits ($n = 1{,}426$) for a period of 18 weeks only when cholera cases were few (61). In a subsequent large-scale field trial in Peru ($n = 9{,}012$), the two-dose WC-rCTB vaccine failed to protect any of the vaccinees (protective efficacy of −4%) during the first year of surveillance (30). A moderate overall protection of 61% was achieved only after a booster dose was delivered 10 months after the second dose. In brief, the results of the Bangladeshi field trial in 1985 have been inappropriately used in a number of publications to justify Dukoral's high protective efficacy of 85% in the early period after vaccination.

CONCLUDING REMARKS ON THE KILLED OCVS SHANCHOL AND DUKORAL

A comparative evaluation of different field trials of the killed OCVs (WC-CTB, WC-rCTB, WC, Shanchol) during the first year after vaccination is shown in Table 6.

Of the two vaccines (Shanchol and Dukoral), Shanchol is preferred because it offers a few operational advantages, such as not requiring a buffer solution for administration, requiring a smaller cold supply chain volume, being applicable to children from 1 year of age (compared to 2 years of age for Dukoral), and being less expensive to produce (35). However, neither Shanchol nor Dukoral appears suitable for cholera control whether it is epidemic or endemic. Biased protective-efficacy results have come out of the trials of these vaccines conducted in areas where cholera is heavily endemic and the adult population was already primed to natural cholera antigens. Children under 5 years of age represent the group most vulnerable to cholera (36), an observation confirmed in a survey during the recent cholera epidemic in Haiti, in which diarrheal disease in children under 5 years of age was a major contributor to pediatric hospitalizations and deaths (98). Shanchol, the preferred vaccine, showed a very poor protective efficacy of 17% in children under 5 years of age; participants of all ages received a modest protective effect of 45% during the first year of surveillance in the Kolkata trial of 2006 (28, 29). Little information is available about the long-term protective efficacy of Dukoral. While a short-term (6-month) case-control trial of two doses of Dukoral in Mozambique in 2004 demonstrated 82% protection in children under 5 years of age (67), a placebo-controlled, double-

TABLE 6 Comparative evaluation of field trials of killed OCVs

Factor	WC-CTB	WC-rCTB	WC-rCTB	WC	WC
Trial location	Bangladesh	Peru	Zanzibar	Bangladesh	India
Yr	1985	1994	2009	1985	2006
Trial method[a]	RCT, DB, PC	RCT, DB, PC	Non-RCT, not DB, not PC	RCT, DB, PC	CRT, DB, PC
Vaccine type	Killed *V. cholerae* O1	Killed *V. cholerae* O1	Killed *V. cholerae* O1	Killed *V. cholerae* O1	Killed *V. cholerae* O1 + O139
Composition/dose	1×10^{11} cells + CTB (1 mg)	1×10^{11} cells + rCTB (1 mg)	1×10^{11} cells + rCTB (1 mg)	1×10^{11} cells	1.75×10^{11} cells
Placebo	Killed *E. coli* K-12	Killed *E. coli* K-12	No placebo	Killed *E. coli* K-12	Killed *E. coli* K-12
No. of doses	3	2[b]	2	3	2
No. of vaccinees	21,141	10,592	23,921	21,137	31,932
% of males	31	46	44	30	50
% of females	69	54	56	70	50
1-yr % PE in:					
<5 yr	38	Negative	Not reported	31	17
All age groups	62	Negative	79	53	45
Reference(s)	24–26	30	65	24–26	28, 29

[a] RCT, randomized controlled trial; DB, double blind; PC, placebo controlled; CRT, cluster-randomized trial.
[b] A third dose of the vaccine was administered after the first two doses were given. In the year of surveillance following administration of the third dose, the protective efficacy (PE) in children <5 years old and in test subjects of all ages were 51 and 61%, respectively (30).

blind, large-scale, two-dose trial of Dukoral in Peru in 1994 produced negative protection during the first year of surveillance (30). The protective efficacy of Dukoral in the Zanzibar trial of 2009 in children under 5 years of age was not reported (65). Because of its poor protective efficacy in children under 5 years of age, a single booster dose of the vaccine every 6 months is recommended for continuous protection (72).

Both the Shanchol and Dukoral vaccines have uncertain compositions. While Shanchol's composition has been inaccurately described in terms of undefined ELISA units, Dukoral includes CTB derived from a classical instead of an El Tor strain. Both of the vaccines are inactivated by heat and formalin treatment, potentially denaturing bacterial protein components (44–47) and reducing their T cell immunogenicity (46). Both Shanchol and Dukoral are two-spaced-dose vaccines, with immunity developing at least 1 week after the last vaccination, reducing their efficacy once an epidemic occurs, as happened recently in Iraq and Zimbabwe (74, 75). Moreover, though the manufacturers of both vaccines claim that they are inexpensive, the cost may be prohibitive in economically strapped regions at risk for cholera. Both of the vaccines comprise formalin-inactivated strains with the possibility of formalin's presence in the final product acting as an allergen to formalin-sensitive people. In summary, factors such as short-term efficacy, poor protection in children under 5 years of age, the necessity for multiple doses, the requirement of a cold supply chain, production cost, and complex logistics of vaccine delivery greatly reduce the suitability of either of these vaccines for endemic or epidemic cholera control in resource-poor settings.

Immunological studies comparing the immune responses induced by WC-rCTB and natural cholera have revealed the reduced and short-lived efficacy of WC-rCTB (79–82). Although these studies were carried out with Dukoral (WC-rCTB), it is likely that similar observations may emerge with the other killed oral vaccine, Shanchol, as protective immunity against cholera is predominantly antibacterial (86, 99). The major difference between Dukoral and Shanchol is that the former contains additional rCTB (60) and the latter has additional *V. cholerae* O139 cells (32). During cholera infection, *V. cholerae* O1 strains, apart from CT, secrete several biologically active products such as neuraminidase, mucinase, collagenase, lipase, and proteinase (100, 101), a few of which are being considered as vaccine candidates (102, 103). A killed cholera vaccine cannot present these factors to the host and hence produces an immune response that is less broad than that induced by natural cholera.

The outcome of a vaccine trial is of great importance for the welfare of people at risk for cholera. The efficacy of a candidate cholera vaccine should be determined by a randomized, double-blind, placebo-controlled trial rather than by less reliable means that are neither placebo controlled nor double blind such as that carried out in the field tests of Dukoral in Zanzibar (65) and Mozambique (67). The trial should include an adequate number of children under 5 years of age, as they represent the group most vulnerable to cholera. Further the vaccine trials should be supervised by independent and impartial monitors with no conflict of interest.

A few laboratories are working on the development of attenuated live *V. cholerae* O1 strains as oral vaccine candidates. This communication has presented an in-depth analysis of the currently available killed OCVs. A detailed discussion of live oral attenuated cholera vaccines, which are not currently available for use and are at different stages of development, is not considered here. Despite extensive research for >100 years, an effective vaccine against cholera has not yet been obtained (73). A single-dose economical vaccine offering a high degree of protection to all age groups in general and to children under 5 years of age in particular is still needed.

REFERENCES

1. **Waldman RJ, Mintz ED, Papowitz HE.** 2013. The cure for cholera—improving access to safe water and sanitation. N. Engl. J. Med. **368:**592–594. http://dx.doi.org/10.1056/NEJMp1214179.
2. **Centers for Disease Control and Prevention.** 2012. Cholera and other *Vibrio* illness surveillance (COVIS) overview, p 2. Centers for Disease Control and Prevention, Atlanta, GA.
3. **Hall RH, Khambaty FM, Kothary MH, Keasler SP, Tall BD.** 1994.

Vibrio cholerae non-O1 serogroup associated with cholera gravis genetically and physiologically resembles O1 E1 Tor cholera strains. Infect. Immun. **62:**3859–3863.
4. **Johnson JA, Salles CA, Panigrahi P, Albert MJ, Wright AC, Johnson RJ, Morris JG, Jr.** 1994. *Vibrio cholerae* O139 synonym Bengal is closely related to *Vibrio cholerae* El Tor but has important differences. Infect. Immun. **62:**2108–2110.
5. **Alam M, Hasan NA, Sadique A, Bhuiyan NA, Ahmed KU, Nusrin S, Nair GB, Siddique AK, Sack RB, Sack DA, Huq A, Colwell RR.** 2006. Seasonal cholera caused by *Vibrio cholerae* serogroups O1 and O139 in the coastal aquatic environment of Bangladesh. Appl. Environ. Microbiol. **72:**4096–4104. http://dx.doi.org/10.1128/AEM.00066-06.
6. **Ramamurthy T, Garg S, Sharma R, Bhattacharya SK, Nair GB, Shimada T, Takeda T, Karasawa T, Kurazano H, Pal A, Takeda Y.** 1993. Emergence of novel strain of *Vibrio cholerae* with epidemic potential in southern and eastern India. Lancet **341:**703–704. http://dx.doi.org/10.1016/0140-6736(93)90480-5.
7. **Asaduzzaman M, Ryan ET, John M, Hang L, Khan AI, Faruque AS, Taylor RK, Calderwood SB, Qadri F.** 2004. The major subunit of the toxin-coregulated pilus TcpA induces mucosal and systemic immunoglobulin A immune responses in patients with cholera caused by *Vibrio cholerae* O1 and O139. Infect. Immun. **72:**4448–4454. http://dx.doi.org/10.1128/IAI.72.8.4448-4454.2004.
8. **Black RE, Levine MM, Clements ML, Young CR, Svennerholm AM, Holmgren J.** 1987. Protective efficacy in humans of killed whole-vibrio oral vaccine with and without the B subunit of cholera toxin. Infect. Immun. **55:**1116–1620.
9. **Kabir S.** 1983. Immmunochemical properties of the major outer membrane protein of *Vibrio cholerae*. Infect. Immun. **39:**452–455.
10. **Majumdar AS, Dutta P, Dutta D, Ghose AC.** 1981. Antibacterial and antitoxin responses in the serum and milk of cholera patients. Infect. Immun. **32:**1–8.
11. **Richardson K, Kaper JB, Levine MM.** 1989. Human immune response to *Vibrio cholerae* O1 whole cells and isolated outer membrane antigens. Infect. Immun. **57:**495–501.
12. **Bhuiyan TR, Lundin SB, Khan AI, Lundgren A, Harris JB, Calderwood SB, Qadri F.** 2009. Cholera caused by *Vibrio cholerae* O1 induces T-cell responses in the circulation. Infect. Immun. **77:**1888–1893. http://dx.doi.org/10.1128/IAI.01101-08.
13. **Harris AM, Bhuiyan MS, Chowdhury F, Khan AI, Hossain A, Kendall EA, Rahman A, LaRocque RC, Wrammert J, Ryan ET, Qadri F, Calderwood SB, Harris JB.** 2009. Antigen-specific memory B-cell responses to *Vibrio cholerae* O1 infection in Bangladesh. Infect. Immun. **77:**3850–3856. http://dx.doi.org/10.1128/IAI.00369-09.
14. **Weil AA, Arifuzzaman M, Bhuiyan TR, LaRocque RC, Harris AM, Kendall EA, Hossain A, Tarique AA, Sheikh A, Chowdhury F, Khan AI, Murshed F, Parker KC, Banerjee KK, Ryan ET, Harris JB, Qadri F, Calderwood SB.** 2009. Memory T-cell responses to *Vibrio cholerae* O1 infection. Infect. Immun. **77:**5090–5096. http://dx.doi.org/10.1128/IAI.00793-09.
15. **Qadri F, Ryan ET, Faruque AS, Ahmed F, Khan AI, Islam MM, Akramuzzaman SM, Sack DA, Calderwood SB.** 2003. Antigen-specific immunoglobulin A antibodies secreted from circulating B cells are an effective marker for recent local immune responses in patients with cholera: comparison to antibody-secreting cell responses and other immunological markers. Infect. Immun. **71:**4808–4814. http://dx.doi.org/10.1128/IAI.71.8.4808-4814.2003.
16. **Svennerholm AM, Jertborn M, Gothefors L, Karim AM, Sack DA, Holmgren J.** 1984. Mucosal antitoxic and antibacterial immunity after cholera disease and after immunization with a combined B subunit-whole cell vaccine. J. Infect. Dis. **149:**884–893. http://dx.doi.org/10.1093/infdis/149.6.884.
17. **Guinée PA, Jansen WH, Gielen H, Rijpkema SG, Peters PW.** 1987. Protective immunity against *Vibrio cholerae* infection in the rabbit. Zentralbl. Bakteriol. Mikrobiol. Hyg. A **266:**552–562.
18. **Kabir S.** 1987. Immunological responses of rabbits to various somatic and secreted antigens of *Vibrio cholerae* after intra-duodenal inoculation. J. Med. Microbiol. **24:**29–40. http://dx.doi.org/10.1099/00222615-24-1-29.
19. **Levine MM, Black RE, Clements ML, Cisneros L, Nalin DR, Young CR.** 1981. Duration of infection-derived immunity to cholera. J. Infect. Dis. **143:**818–820. http://dx.doi.org/10.1093/infdis/143.6.818.
20. **Koch R.** 1883. Der zweite Bericht der deutschen Cholera-Commission. Dtsch. Med. Wochenschr. **9:**743–744. (In German.)
21. **Bornside GH.** 1982. Waldemar Haffkine's cholera vaccines and the Ferran-Haffkine priority dispute. J. Hist. Med. Allied Sci. **37:**399–422.
22. **Bhadra RK, Das S, Nair GB.** 2010. Efficacy of cholera vaccines, p 18–20. *In* Bhadra RK, Das S, Nair GB (ed), The immunological basis for immunization series—module 14: cholera. World Health Organization, Geneva, Switzerland.
23. **Bhattacharya SK, Sur D, Ali M, Kanungo S, You YA, Manna B, Sah B, Niyogi SK, Park JK, Sarkar B, Puri MK, Kim DR, Deen JL, Holmgren J, Carbis R, Dhingra MS, Donner A, Nair GB, Lopez AL, Wierzba TF, Clemens JD.** 2013. 5 year efficacy of a bivalent killed whole-cell oral cholera vaccine in Kolkata, India: a cluster-randomised, double-blind, placebo-controlled trial. Lancet Infect. Dis. **13:**1050–1056. http://dx.doi.org/10.1016/S1473-3099(13)70273-1.
24. **Clemens JD, Sack DA, Harris JR, Chakraborty J, Khan MR, Stanton BF, Kay BA, Khan MU, Yunus M, Atkinson W, Svennerhom AM, Holmgren J.** 1986. Field trial of oral cholera vaccines in Bangladesh. Lancet **ii:**124–127.
25. **Clemens JD, Harris JR, Sack DA, Chakraborty J, Ahmed F, Stanton BF, Khan MU, Kay BA, Huda N, Khan MR, Yunus M, Rao RM, Svennerholm AM, Holmgren J.** 1988. Field trial of oral cholera vaccines in Bangladesh: results of one year of follow-up. J. Infect. Dis. **158:**60–69. http://dx.doi.org/10.1093/infdis/158.1.60.
26. **Clemens JD, Sack DA, Harris JR, Van Loon F, Chakraborty J, Ahmed F, Rao MR, Khan MR, Yunus M, Huda N, Stanton BF, Kay BA, Walter S, Eeckels R, Svennerholm AM, Holmgren J.** 1990. Field trial of oral cholera vaccines in Bangladesh: results from three-year follow-up. Lancet **335:**270–273. http://dx.doi.org/10.1016/0140-6736(90)90080-O.
27. **Richie EE, Punjabi NH, Sidharta YY, Peetosutan KK, Sukandar MM, Wasserman SS, Lesmana MM, Wangsasaputra FF, Pandam SS, Levine MM, O'Hanley PP, Cryz SJ, Simanjuntak CH.** 2000. Efficacy trial of single-dose live oral cholera vaccine CVD 103-HgR in North Jakarta, Indonesia, a cholera-endemic area. Vaccine **18:**2399–2410. http://dx.doi.org/10.1016/S0264-410X(00)00006-2.
28. **Sur D, Lopez AL, Kanungo S, Paisley A, Manna B, Ali M, Niyogi SK, Park JK, Sarkar B, Puri MK, Kim DR, Deen JL, Holmgren J, Carbis R, Rao R, Nguyen TV, Donner A, Ganguly NK, Nair GB, Bhattacharya SK, Clemens JD.** 2009. Efficacy and safety of a modified killed-whole-cell oral cholera vaccine in India: an interim analysis of a cluster-randomised, double-blind, placebo-controlled trial. Lancet **374:**1694–1702. http://dx.doi.org/10.1016/S0140-6736(09)61297-6.
29. **Sur D, Kanungo S, Sah B, Manna B, Ali M, Paisley AM, Niyogi SK, Park JK, Sarkar B, Puri MK, Kim DR, Deen JL, Holmgren J, Carbis R, Rao R, Nguyen TV, Han SH, Attridge S, Donner A, Ganguly NK, Bhattacharya SK, Nair GB, Clemens JD, Lopez AL.** 2011. Efficacy of a low-cost, inactivated whole-cell oral cholera vaccine: results from 3 years of follow-up of a randomized, controlled trial. PLoS Negl. Trop. Dis. **5**(10):e1289. http://dx.doi.org/10.1371/journal.pntd.0001289.
30. **Taylor DN, Cárdenas V, Sanchez JL, Bégué RE, Gilman R, Bautista C, Perez J, Puga R, Gaillour A, Meza R, Echeverria P, Sadoff J.** 2000. Two-year study of the protective efficacy of the oral whole cell plus recombinant B subunit cholera vaccine in Peru. J. Infect. Dis. **181:**1667–1673. http://dx.doi.org/10.1086/315462.
31. **World Health Organization.** 2005. Oral cholera vaccine stockpile for emergency response. World Health Organization, Geneva, Switzerland.
32. **World Health Organization.** 2011. Shanchol, inactivated oral cholera vaccine. World Health Organization, Geneva, Switzerland.
33. **Ivers LC, Teng JE, Lascher J, Raymond M, Weigel J, Victor N, Jerome JG, Hilaire IJ, Almazor CP, Ternier R, Cadet J, Francois J, Guillaume FD, Farmer PE.** 2013. Use of oral cholera vaccine in Haiti: a rural demonstration project. Am. J. Trop. Med. Hyg. **89:**617–624. http://dx.doi.org/10.4269/ajtmh.13-0183.
34. **Rouzier V, Severe K, Juste MA, Peck M, Perodin C, Severe P, Deschamps MM, Verdier RI, Prince S, Francois J, Cadet JR, Guillaume FD, Wright PF, Pape JW.** 2013. Cholera vaccination in urban Haiti. Am. J. Trop. Med. Hyg. **89:**671–681. http://dx.doi.org/10.4269/ajtmh.13-0171.
35. **Vicari AS, Ruiz-Matus C, de Quadros C, Andrus JK.** 2013. Development of a cholera vaccination policy on the island of Hispaniola, 2010-2013. Am. J. Trop. Med. Hyg. **89:**682–687. http://dx.doi.org/10.4269/ajtmh.13-0200.

36. **Anonymous.** 2013. Cholera toolkit, p 6. United Nations Children's Fund, New York, NY.
37. **Anh DD, Canh do, Lopez GAL, Thiem VD, Long PT, Son NH, Deen J, von Seidlein L, Carbis R, Han SH, Shin SH, Attridge S, Holmgren J, Clemens J.** 2007. Safety and immunogenicity of a reformulated Vietnamese bivalent killed, whole-cell, oral cholera vaccine in adults. Vaccine **25:**1149–1155. http://dx.doi.org/10.1016/j.vaccine.2006.09.049.
38. **Barzilay EJ, Schaad N, Magloire R, Mung KS, Boncy J, Dahourou GA, Mintz ED, Steenland MW, Vertefeuille JF, Tappero JW.** 2013. Cholera surveillance during the Haiti epidemic—the first 2 years. N. Engl. J. Med. **368:**599–609. http://dx.doi.org/10.1056/NEJMoa1204927.
39. **Pan American Health Organization.** 2014. WHO Epidemiological update cholera 20 March 2014. World Health Organization, Geneva, Switzerland.
40. **Anonymous.** 2013. XXI Meeting of the Technical Advisory Group on Vaccine-Preventable Diseases: Vaccination: A Shared Responsibility. 3 to 5 July 2013, Quito, Ecuador, p 63. World Health Organization, Geneva, Switzerland.
41. **Anonymous.** 2008. A randomized controlled trial of the bivalent killed whole cell oral cholera vaccine in eastern Kolkata, West Bengal, India. Protocol version 3.0, section 6.1, study agents (vaccine and placebo), p 17. National Institute of Cholera and Enteric Diseases, Kolkata, India.
42. **Chatterjee SN, Chaudhuri K.** 2003. Lipopolysaccharides of *Vibrio cholerae*. I. Physical and chemical characterization. Biochim. Biophys. Acta **1639:**65–79. http://dx.doi.org/10.1016/j.bbadis.2003.08.004.
43. **Weintraub A, Widmalm G, Jansson PE, Jansson M, Hultenby K, Albert MJ.** 1994. *Vibrio cholerae* O139 Bengal possesses a capsular polysaccharide which may confer increased virulence. Microb. Pathog. **16:**235–241. http://dx.doi.org/10.1006/mpat.1994.1024.
44. **Kabir S.** 1989. Antigenic analysis of *Vibrio cholerae* O1 by crossed immunoelectrophoresis. Zentralbl. Bakteriol. Mikrobiol. Hyg. A **270:**361–372.
45. **Yamamoto T.** 2000. Current status of cholera and rise of novel mucosal vaccine. Jpn. J. Infect. Dis. **53:**181–188.
46. **di Tommaso A, de Magistris MT, Bugnoli M, Marsili I, Rappuoli R, Abrignani S.** 1994. Formaldehyde treatment of proteins can constrain presentation to T cells by limiting antigen processing. Infect. Immun. **62:**1830–1834.
47. **Metz B, Kersten GF, Hoogerhout P, Brugghe HF, Timmermans HA, de Jong A, Meiring H, ten Hove J, Hennink WE, Crommelin DJ, Jiskoot W.** 2004. Identification of formaldehyde-induced modifications in proteins: reactions with model peptides. J. Biol. Chem. **279:**6235–6243. http://dx.doi.org/10.1074/jbc.M310752200.
48. **Islam MS, Mahmud ZH, Ansaruzzaman M, Faruque SM, Talukder KA, Qadri F, Alam M, Islam S, Bardhan PK, Mazumder RN, Khan AI, Ahmed S, Iqbal A, Chitsatso O, Mudzori J, Patel S, Midzi SM, Charimari L, Endtz HP, Cravioto A.** 2011. Phenotypic, genotypic, and antibiotic sensitivity patterns of strains isolated from the cholera epidemic in Zimbabwe. J. Clin. Microbiol. **49:**2325–2327. http://dx.doi.org/10.1128/JCM.00432-11.
49. **Mahalanabis D, Lopez AL, Sur D, Deen J, Manna B, Kanungo S, von Seidlein L, Carbis R, Han SH, Shin SH, Attridge S, Rao R, Holmgren J, Clemens J, Bhattacharya SK.** 2008. A randomized, placebo-controlled trial of the bivalent killed, whole-cell, oral cholera vaccine in adults and children in a cholera endemic area in Kolkata, India. PLoS One **3**(6):e2323. http://dx.doi.org/10.1371/journal.pone.0002323.
50. **Anonymous.** 2009. Zimbabwe cholera control guides, 3rd edition, p 8. World Health Organization, Geneva, Switzerland.
51. **Anonymous.** 1967. WHO Expert Committee on Cholera. Second report. World Health Organ. Tech. Rep. Ser. **352:**3–28.
52. **Finkelstein RA.** 1985. Microbial toxins and diarrhoeal diseases. Ciba Found. Symp. **112:**257.
53. **Mosley WH, Aziz KM, Rahman AS, Chowdhury AK, Ahmed A.** 1973. Field trials of monovalent Ogawa and Inaba cholera vaccines in rural Bangladesh—three years of observation. Bull. World Health Organ. **49:**381–387.
54. **Pal SC, Deb BC, Sen Gupta PG, De SP, Sircar BK, Sen D, Sikdar SN.** 1980. A controlled field trial of an aluminium phosphate-adsorbed cholera vaccine in Calcutta. Bull. World Health Organ. **58:**741–745.
55. **Saroso JS, Bahrawi W, Witjaksono H, Budiarso RL, Brotowasisto Bencić Z, Dewitt WE, Gomez CZ.** 1978. A controlled field trial of plain and aluminium hydroxide-adsorbed cholera vaccines in Surabaya, Indonesia, during 1973-75. Bull. World Health Organ. **56:**619–627.
56. **Svennerholm AM, Holmgren J.** 1976. Synergistic protective effect in rabbits of immunization with *Vibrio cholerae* lipopolysaccharide and toxin/toxoid. Infect. Immun. **13:**735–740.
57. **van Loon FP, Clemens JD, Chakraborty J, Rao MR, Kay BA, Sack DA, Yunus M, Ali M, Svennerholm AM, Holmgren J.** 1996. Field trial of inactivated oral cholera vaccines in Bangladesh: results from 5 years of follow-up. Vaccine **14:**162–166. http://dx.doi.org/10.1016/0264-410X(95)00122-H.
58. **Sack DA, Clemens JD, Huda S, Harris JR, Khan MR, Chakraborty J, Yunus M, Gomes J, Siddique O, Ahmed F, Kay BA, van Loon FPL, Rao MR, Svennerholm AM, Holmgren J.** 1991. Antibody responses after immunization with killed oral cholera vaccines during the 1985 vaccine field trial in Bangladesh. J. Infect. Dis. **164:**407–411. http://dx.doi.org/10.1093/infdis/164.2.407.
59. **Sanchez J, Holmgren J.** 1989. Recombinant system for overexpression of cholera toxin B subunit in *Vibrio cholerae* as a basis for vaccine development. Proc. Natl. Acad. Sci. U. S. A. **86:**481–485. http://dx.doi.org/10.1073/pnas.86.2.481.
60. **Anonymous.** 2007. Dukoral monograph. Läkemedelsverket (Medical Products Agency Sweden), Uppsala, Sweden. (In Swedish.)
61. **Sanchez JL, Vasquez B, Begue RE, Meza R, Castellares G, Cabezas C, Watts DM, Svennerholm AM, Sadoff JC, Taylor DN.** 1994. Protective efficacy of oral whole-cell/recombinant-B-subunit cholera vaccine in Peruvian military recruits. Lancet **344:**1273–1276. http://dx.doi.org/10.1016/S0140-6736(94)90755-2.
62. **Taylor DN, Sanchez J, Cardenas V, Gilman RE, Sadoff J.** 2001. Reply to misleading negative findings in a field trial of killed, oral cholera vaccine in Peru. J. Infect. Dis. **183:**1308–1309. http://dx.doi.org/10.1086/319682.
63. **Clemens JD, Sack DA, Ivanoff B.** 2001. Misleading negative findings in a field trial of killed, oral cholera vaccine in Peru. J. Infect. Dis. **183:**1306–1308. http://dx.doi.org/10.1086/319673.
64. **Sinclair D, Abba K, Zaman K, Qadri F, Graves PM.** 2011. Oral vaccines for preventing cholera. Cochrane Database Syst. Rev. **16**(3)**:**CD008603. http://dx.doi.org/10.1002/14651858.CD008603.pub2.
65. **Khatib AM, Ali M, von Seidlein L, Kim DR, Hashim R, Reyburn R, Ley B, Thriemer K, Enwere G, Hutubessy R, Aguado MT, Kieny MP, Lopez AL, Wierzba TF, Ali SM, Saleh AA, Mukhopadhyay AK, Clemens J, Jiddawi MS, Deen J.** 2012. Effectiveness of an oral cholera vaccine in Zanzibar: findings from a mass vaccination campaign and observational cohort study. Lancet Infect. Dis. **12:**837–844. http://dx.doi.org/10.1016/S1473-3099(12)70196-2.
66. **Clemens JD, Sack DA, Harris JR, Chakraborty J, Neogy PK, Stanton B, Huda N, Khan MU, Kay BA, Khan MR, Ansaruzzaman AM, Yunus M, Rao MR, Svennerholm AM, Holmgren J.** 1988. Cross-protection by B subunit-whole cell cholera vaccine against diarrhea associated with heat-labile toxin-producing enterotoxigenic *Escherichia coli*: results of a large-scale field trial. J. Infect. Dis. **158:**372–377. http://dx.doi.org/10.1093/infdis/158.2.372.
67. **Lucas ME, Deen JL, von Seidlein L, Wang XY, Ampuero J, Puri M, Ali M, Ansaruzzaman M, Amos J, Macuamule A, Cavailler P, Guerin PJ, Mahoudeau C, Kahozi-Sangwa P, Chaignat CL, Barreto A, Songane FF, Clemens JD.** 2005. Effectiveness of mass oral cholera vaccination in Beira, Mozambique. N. Engl. J. Med. **352:**757–767. http://dx.doi.org/10.1056/NEJMoa043323.
68. **Finkelstein RA.** 1995. Why do we not yet have a suitable vaccine against cholera? Adv. Exp. Med. Biol. **371B:**1633–1640.
69. **Anonymous.** 2014. Dukoral product information. Crucell Sweden AB, Solna, Sweden.
70. **Anonymous.** 2004. Annex 3. Guidelines for the production of inactivated oral cholera vaccines, p 129–149. WHO Expert Committee on Biological Standardization: fifty-second report, 26 to 30 November 2001. World Health Organization, Geneva, Switzerland. http://www.who.int/biologicals/publications/trs/areas/vaccines/cholera/129-149.pdf.
71. **Marchlewicz BA, Finkelstein RA.** 1983. Immunological differences among the cholera/coli family of enterotoxins. Diagn. Microbiol. Infect. Dis. **1:**129–138. http://dx.doi.org/10.1016/0732-8893(83)90042-1.
72. **Anonymous.** 2011. Dukoral product information. European Medicines Agency, London, United Kingdom. http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/000476/WC500037570.pdf.
73. **Kabir S.** 2007. Cholera vaccines. Lancet Infect. Dis. **7:**176–178. http://dx.doi.org/10.1016/S1473-3099(07)70032-4.

74. **Anonymous.** 2007. Position paper on cholera vaccine use in Iraq, 05 October. World Health Organization, Geneva, Switzerland.
75. **Anonymous.** 2008. Global alert and response, cholera in Zimbabwe. World Health Organization, Geneva, Switzerland.
76. **Anonymous.** 2008. Dukoral product information. SBL Vaccin AB, Solna, Sweden.
77. **Sanchez JL, Hayashi KE, Kruger HF, Meza R, English CK, Vidal W, Svennerholm AM, Taylor DN.** 1995. Immunological response to *Vibrio cholerae* O1 infection and an oral cholera vaccine among Peruvians. Trans. R. Soc. Trop. Med. Hyg. **89:**542–545. http://dx.doi.org/10.1016/0035-9203(95)90103-5.
78. **Ortigão-de-Sampaio MB, Shattock RJ, Hayes P, Griffin GE, Linhares-de-Carvalho MI, Ponce de Leon A, Lewis DJ, Castello-Branco LR.** 1998. Increase in plasma viral load after oral cholera immunization of HIV-infected subjects. AIDS **12:**F145–F150. http://dx.doi.org/10.1097/00002030-199814000-00001.
79. **Alam MM, Riyadh MA, Fatema K, Rahman MA, Akhtar N, Ahmed T, Chowdhury MI, Chowdhury F, Calderwood SB, Harris JB, Ryan ET, Qadri F.** 2011. Antigen-specific memory B-cell responses in Bangladeshi adults after one- or two-dose oral killed cholera vaccination and comparison with responses in patients with naturally acquired cholera. Clin. Vaccine Immunol. **18:**844–850. http://dx.doi.org/10.1128/CVI.00562-10.
80. **Arifuzzaman M, Rashu R, Leung DT, Hosen MI, Bhuiyan TR, Bhuiyan MS, Rahman MA, Khanam F, Saha A, Charles RC, LaRocque RC, Weil AA, Clements JD, Holmes RK, Calderwood SB, Harris JB, Ryan ET, Qadri F.** 2012. Antigen-specific memory T cell responses after vaccination with an oral killed cholera vaccine in Bangladeshi children and comparison to responses in patients with naturally acquired cholera. Clin. Vaccine Immunol. **19:**1304–1311. http://dx.doi.org/10.1128/CVI.00196-12.
81. **Leung DT, Rahman MA, Mohasin M, Patel SM, Aktar A, Khanam F, Uddin T, Riyadh MA, Saha A, Alam MM, Chowdhury F, Khan AI, Charles R, LaRocque R, Harris JB, Calderwood SB, Qadri F, Ryan ET.** 2012. Memory B cell and other immune responses in children receiving two doses of an oral killed cholera vaccine compared to responses following cholera infection in Bangladesh. Clin. Vaccine Immunol. **19:**690–698. http://dx.doi.org/10.1128/CVI.05615-11.
82. **Pasetti MF, Levine MM.** 2012. Insights from natural infection-derived immunity to cholera instruct vaccine efforts. Clin. Vaccine Immunol. **19:**1707–1711. http://dx.doi.org/10.1128/CVI.00543-12.
83. **Mosley WH, McCormack WM, Ahmed A, Chowdhury AK, Barui RK.** 1969. Report of the 1966-67 cholera vaccine field trial in rural East Pakistan. 2. Results of the serological surveys in the study population—the relationship of case rate to antibody titre and an estimate of the inapparent infection rate with *Vibrio cholerae*. Bull. World Health Organ. **40:**187–197.
84. **Northrup RS, Fauci AS.** 1972. Adjuvant effect of cholera enterotoxin on the immune response of the mouse to sheep red blood cell. J. Infect. Dis. **125:**672–673. http://dx.doi.org/10.1093/infdis/125.6.672.
85. **Hill DR, Ford L, Lalloo DG.** 2006. Oral cholera vaccines: use in clinical practice. Lancet Infect. Dis. **6:**361–373. http://dx.doi.org/10.1016/S1473-3099(06)70494-7.
86. **Kabir S.** 2005. Cholera vaccines: current status and problems. Rev. Med. Microbiol. **16:**101–116. http://dx.doi.org/10.1097/01.revmedmi.0000174307.33651.81.
87. **Jertborn M, Svennerholm AM, Holmgren J.** 1992. Safety and immunogenicity of an oral recombinant cholera B subunit-whole cell vaccine in Swedish volunteers. Vaccine **10:**130–132. http://dx.doi.org/10.1016/0264-410X(92)90030-N.
88. **Jertborn M, Svennerholm AM, Holmgren J.** 1994. Immunological memory after immunization with oral cholera B subunit—the whole-cell vaccine in Swedish volunteers. Vaccine **12:**1078–1082. http://dx.doi.org/10.1016/0264-410X(94)90176-7.
89. **Masuet Aumatell C, Ramon Torrell JM, Zuckerman JN.** 2011. Review of oral cholera vaccines: efficacy in young children. Infect. Drug Resist. **4:**155–160. http://dx.doi.org/10.2147/IDR.S10339.
90. **Anonymous.** 2010. Cholera vaccines: WHO position paper. Wkly. Epidemiol. Rec. **85:**117–128.
91. **Girard MP, Steele D, Chaignat C-L, Kieny MP.** 2006. A review of vaccine research and development: human enteric infections. Vaccine **24:**2732–2750. http://dx.doi.org/10.1016/j.vaccine.2005.10.014.
92. **Lopez AL, Clemens JD, Dean J, Jodar L.** 2008. Cholera vaccines for the developing world. Hum. Vaccines **4:**165–169. http://dx.doi.org/10.4161/hv.4.2.5122.
93. **López-Gigosos RM, Plaza E, Díez-Díaz RM, Calvo MJ.** 2011. Vaccination strategies to combat an infectious globe: oral cholera vaccines. J. Glob Infect. Dis. **3:**56–62. http://dx.doi.org/10.4103/0974-777X.77297.
94. **Pastor M, Pedraz JL, Esquisabel A.** 2013. The state-of-the art of approved and under-development cholera vaccines. Vaccine **31:**4069–4078. http://dx.doi.org/10.1016/j.vaccine.2013.06.096.
95. **Qadri F, Bhuiyan TR, Sack DA, Svennerholm AM.** 2013. Immune responses and protection in children in developing countries induced by oral vaccines. Vaccine **31:**452–460. http://dx.doi.org/10.1016/j.vaccine.2012.11.012.
96. **Sack DA, Sack RB, Nair GB, Siddique AK.** 2004. Cholera. Lancet **363:**223–233. http://dx.doi.org/10.1016/S0140-6736(03)15328-7.
97. **Anonymous.** 2005. Dukoral, det drickbara resevaccinet. SBL Vaccines, Stockholm, Sweden. (In Swedish.)
98. **Derby KS, Lucien MA, Leshem E, Steenland MW, Juin S, Joseph GA, Katz MA.** 2014. Hospitalizations and deaths caused by diarrhea in children five years old and younger at four hospitals in Haiti, 2010-2012. Am. J. Trop. Med. Hyg. **90:**291–293. http://dx.doi.org/10.4269/ajtmh.13-0370.
99. **Levine MM, Nalin DR, Craig JP, Hoover D, Bergquist EJ, Waterman D, Holley HP, Hornick RB, Pierce NP, Libonati JP.** 1979. Immunity of cholera in man: relative role of antibacterial versus antitoxic immunity. Trans. R. Soc. Trop. Med. Hyg. **73:**3–9. http://dx.doi.org/10.1016/0035-9203(79)90119-6.
100. **Kabir S, Ahmed N, Ali S.** 1984. Neuraminidase production by *Vibrio cholerae* O1 and other diarrheagenic bacteria. Infect. Immun. **44:**747–749.
101. **Shrivastava DL.** 1964. Enzymes in *Vibrio cholerae*. Indian J. Med. Res. **52:**801–808.
102. **Stewart-Tull DE, Bleakley CR, Galloway TS.** 2004. Characteristics of *Vibrio cholerae* proteinases: potential, candidate vaccine antigens. Vaccine **22:**3026–3034. http://dx.doi.org/10.1016/j.vaccine.2004.02.012.
103. **Stewart-Tull DE, Lucas C, Bleakley CR.** 2004. Experimental immunisation and protection of guinea pigs with *Vibrio cholerae* toxoid and mucinases, neuraminidase and proteinase. Vaccine **22:**2137–2145. http://dx.doi.org/10.1016/j.vaccine.2003.12.004.